Biosynthesis and Catabolism of Caffeine in Low-Caffeine-Containing Species of *Coffea*

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Leaves of *Coffea salvatrix*, *Coffea eugenioides*, and *C. bengalensis* contain ~3−7-fold lower levels of caffeine than those of *Coffea arabica*. There was more extensive biosynthesis of caffeine from [8-^{14}C]-adenine in young leaves of *C. arabica* than in *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*. Degradation of [8-^{14}C]caffeine, which is negligible in leaves of *C. arabica*, was also very slow in *C. salvatrix* and *C. bengalensis*. In contrast, [8-^{14}C]caffeine was catabolized rapidly by young and mature leaves of *C. eugenioides* primarily by a caffeine → theophylline → 3-methylxanthine → xanthine → uric acid → allantoin → allantoic acid → urea → CO_2 + NH_3 pathway. These results indicate that the low caffeine accumulation in *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* is a consequence of a slow rate of caffeine biosynthesis, whereas rapid degradation of caffeine also contributes to the low endogenous caffeine pool in *C. eugenioides*. The genes that regulate caffeine accumulation appear to be those encoding *N*-methyltransferase and caffeine (7-*N*) demethylase activities. The diversity of caffeine catabolism observed in *C. arabica*, *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*, other species of *Coffea*, and *Camellia sinensis* is discussed.

Keywords: *Caffeine; biosynthesis; catabolism; Coffea arabica; Coffea salvatrix; Coffea eugenioides; Coffea bengalensis*

INTRODUCTION

Coffee, produced primarily in Central and South America and Africa, is one of the world's most valuable agricultural products because of its widespread use as a beverage. Fruits of *Coffea arabica* and *Coffea canephora* are the raw materials for the production of Arabica and Robusta coffee. These coffee beans contain relatively high concentrations of caffeine, which is perceived by some members of the general public as having adverse effects on health (Mazzafera et al., 1991). As a consequence, there is an increasing demand for decaffeinated coffee, which is produced by solvent extraction or, more recently, supercritical fluid extraction with CO_2. An alternative source of decaffeinated coffee would be the use of *Coffea* species that contain much lower levels of caffeine than *C. arabica* or *C. canephora*. A number of such species exist, but they either produce few fruits or the beans yield a poor quality beverage and are, therefore, intrinsically unsuitable for commercial development. Nor is a breeding program to transfer the low-caffeine trait to *C. arabica* a straightforward proposition because *C. arabica* and *C. canephora* are polyploid, whereas other *Coffea* species are diploid. Under these circumstances, genetic engineering to produce transgenic caffeine-deficient *C. arabica* plants may be a more practical long-term proposition than a breeding program (Mazzafera et al., 1991; Crozier, 1997). The key genes in this regard are those encoding the *N*-methyltransferases associated with caffeine biosynthesis (see Figure 1) and the *N*-demethylases that catabolize caffeine. Caffeine-deficient transgenic coffee could be produced by expression of the gene encoding the appropriate demethylase or alternatively through the antisense expression of an *N*-methyltransferase gene. For this approach to be taken, detailed information is first required on the enzymes and genes controlling key steps in the biosynthesis and/or catabolism of caffeine.

There is much evidence that caffeine and other purine alkaloids are synthesized in *C. arabica* from purine nucleotides, primarily via the pathway illustrated in Figure 1. Xanthosine is metabolized to 7-methylxanthosine, which is the initial step in the caffeine biosynthesis pathway in which theobromine (3,7-dimethylxanthine) is the immediate precursor of caffeine (Suzuki et al., 1992; Ashihara and Crozier, 1999). There is a report of an alternative entry in the pathway that involves direct conversion of XMP to 7-methyl-XMP (Schulthess et al., 1996). Caffeine biosynthesis occurs in both fruits and leaves of coffee. However, as they are obtained more easily, especially in non-coffee-producing countries, most research has been carried out with leaves. Caffeine biosynthesis is especially active in young leaves of *C. arabica* and declines as the leaves age (Fujimori and Ashihara, 1994; Ashihara et al., 1996a). Caffeine accumulates in *C. arabica* due to extremely slow catabolism to theophylline (1,7-dimethylxanthine) (Ashihara et al., 1996b). To investigate the mechanisms that regulate the accumulation of purine alkaloids, the biosynthesis and catabolism of caffeine have been compared in leaves of *C. arabica* and three

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10.1021/jf981209n CCC: $18.00
Published on Web 07/15/1999

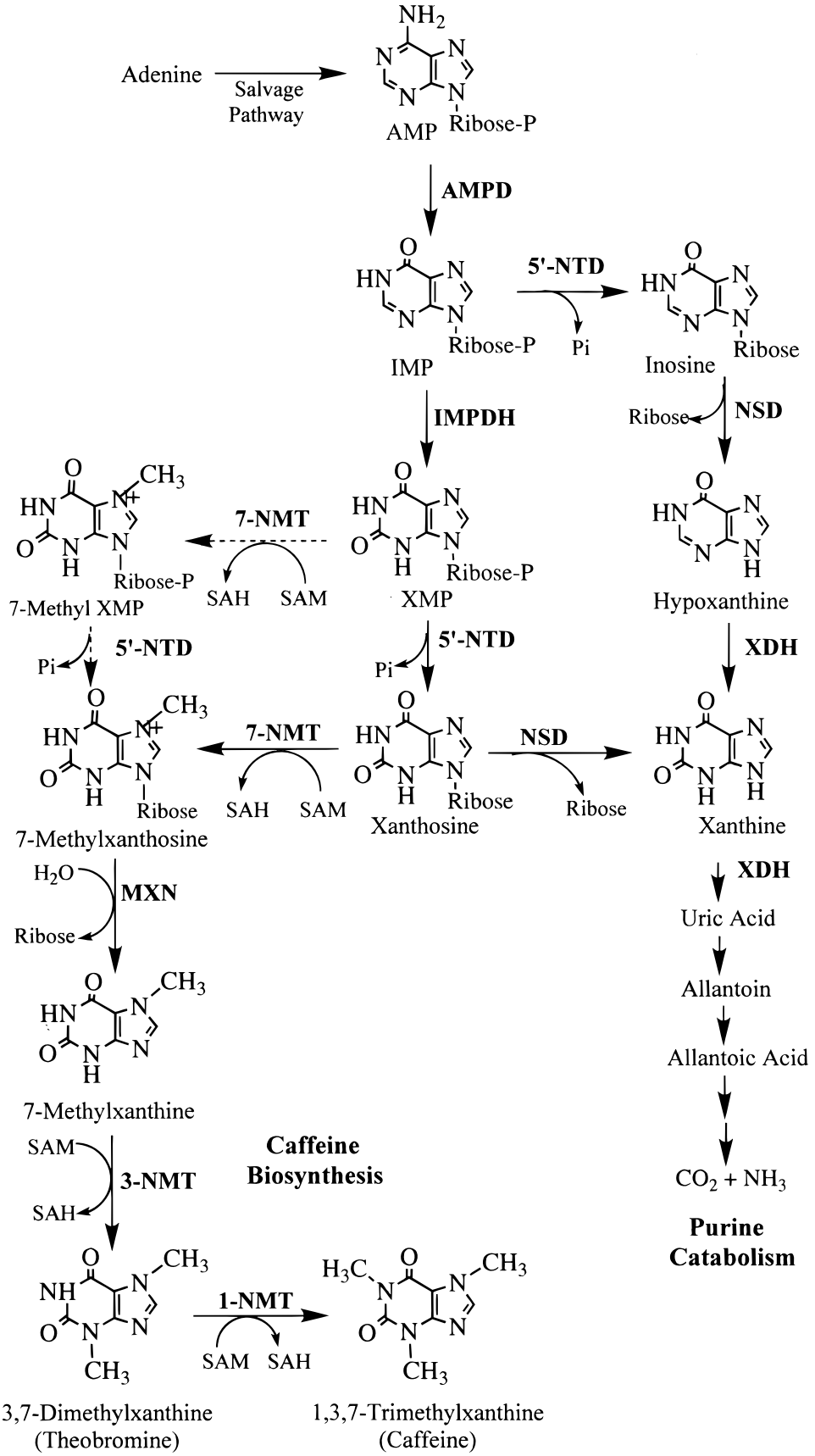

Figure 1. Caffeine biosynthesis and purine catabolic pathways operating in leaves of *C. arabica*. Dotted arrows indicate alternative pathway proposed by Schulthess et al. (1996). Enzymes: AMP deaminase (AMPD); IMP dehydrogenase; (IMPDH) 5′-nucleotidase (5′-NTD); (5) SAM:xanthosine *N-7* methyltransferase (7-NMT); 7-methylxanthosine nucleosidase (MXN); SAM:7-methylxanthine *N-3* methyl transferase (3-NMT); SAM:theobromine *N-1* methyltransferase (1-NMT); xanthosine nucleosidase (NSD); xanthine dehydrogenase (XDH). In tea leaves, the 3-NMT and 1-NMT reactions are catalyzed by a single enzyme called caffeine synthase, which has been purified to homogeneity (Kato et al., 1999).

low-caffeine-containing species of coffee, *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*.

MATERIALS AND METHODS

Plant Material. Seeds of *C. arabica*, *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* were obtained from the Instituto Agronómico, Campinas, São Paulo, Brazil. The leaves used in this study were from 2-year old plants growing under a natural photoperiod in a greenhouse at the University of Glasgow. Young leaves were the most recently emerged primary leaf; mature leaves comprised the fully expanded, second and third leaves below the apex. Typical leaf size and fresh weight of each *Coffea* leaves were as follows: *C. salvatrix* (young) 30 mm long, 12 mm wide, fresh weight (fw) = 80 mg; (mature) 170 mm long, 75 mm wide, fw = 1800 mg; *C. eugenioides* (young) 32 mm long, 12 mm wide, fw = 50 mg; (mature) 90 mm long, 42 mm wide, fw = 450 mg; *C. bengalensis* (young) 50 mm long, 17 mm wide, fw = 70 mg; (mature) 180 mm long, 85 mm wide, fw = 1300 mg; *C. arabica* (young) 20 mm long, 7 mm wide, fw = 20 mg; (mature) 160 mm long, 70 mm wide, fw = 1200 mg.

Radiochemicals. [8-^{14}C]Caffeine (specific activity = 2.07 MBq μmol^{-1}) and [8-^{14}C]adenine (1.96 MBq μmol^{-1}) were purchased from Moravek Biochemicals Inc., Brea, CA. [8-^{14}C]-Theophylline (2.04 MBq μmol^{-1}) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO.

Metabolism of Radiolabeled Purine Derivatives. Segments of *Coffea* leaves (~5 mm strips of young leaves; ~5 mm × 5 mm for mature leaves) were incubated in 2 mL of medium, comprising 30 mM potassium phosphate buffer, pH 5.6, 10 mM sucrose, and a radiolabeled substrate (37 kBq), in a 30 mL Erlenmeyer flask, in a shaking water bath for 18 h at 27 °C. The Erlenmeyer flask had a center well containing a small glass tube into which was inserted a piece of filter paper wetted with 0.1 mL of a 20% potassium hydroxide solution. In pulse−chase experiments leaves were incubated with ^{14}C-labeled substrate (74 kBq) for 4 h, at which point the radiolabel was removed and the incubation continued for a further 20 h.

At the end of the incubation period the glass tube and filter paper from the center well were transferred to a 50 mL flask containing 10 mL of distilled water and, after thorough shaking, radioactivity in a 1 mL aliquot was determined by liquid scintillation counting using ACS II scintillant (Amersham International plc) to determine the amount of $^{14}CO_2$ released during the metabolism period. The leaf segments were separated from the incubation medium by filtering through a tea strainer, washed with 50 mL distilled water then mixed with 5 mL of extraction medium, comprising 80% methanol in 20 mM sodium diethyldithiocarbamate, and ground using a pestle and mortar in ice. The homogenate was centrifuged at 12000*g* for 5 min. The pellet was resuspended in 5 mL of extraction medium and recentrifuged. The two supernatants, containing the methanol-soluble metabolites, were pooled and reduced to dryness in vacuo. The dry material was redissolved in 0.5 mL of 50% methanol, and aliquots were analyzed by TLC and HPLC.

Thin-Layer Chromatography of Radiolabeled Metabolites. The methanol-soluble metabolites were analyzed by TLC using sheets of microcrystalline cellulose as described in previous papers (Ashihara et al., 1997; Ito and Ashihara, 1999). The solvent system used was *n*-butanol/acetic acid/water (4:1:2, v/v). The location and quantification of ^{14}C-labeled compounds on the TLC sheets were determined using a Bio-Imaging Analyzer, Type FLA-2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

High-Performance Liquid Chromatography of Endogenous Purine Alkaloids and Radiolabeled Metabolites. HPLC was carried out as described by Ashihara et al. (1996b, 1997). In the present study, a Shimadzu LC-10A HPLC system was used with a 250 × 4.6 mm i.d. ferruleless column (Capital HPLC Ltd., Broxburn, U.K.) packed in house with a 5-μm ODS Hypersil support (Shandon plc, Runcorn, Cheshire, U.K.). The mobile phase flow rate was 1 mL min^{-1}, and samples were analyzed using a 25 min gradient of 0−40% methanol in 50 mM sodium acetate, pH 5.0, which separates 11 different purine derivatives and is also able to resolve ^{14}C-labeled uric acid, allantoin, and allantoic acid (Ashihara et al., 1996a). The absorbance at 270 nm and subsequent radioactivity detection were monitored using a Shimadzu SPD-10A UV−vis monitor and a Raytest radioanalyzer, Model Ramona 2000 (Raytest Isotopenmessgerate Gmbh, Straubenhardt, Germany).

Table 1. Caffeine Content in Young and Mature Coffee Leaves[a]

species	young leaves	mature leaves
C. arabica cv. Kent	7.1 ± 2.6 (100)	2.1 ± 0.34 (100)
C. salvatrix	0.92 ± 0.08 (13)	0.30 ± 0.01 (14)
C. eugenioides	1.3 ± 0.11 (19)	0.37 ± 0.03 (18)
C. bengalensis	1.2 ± 0.01 (17)	0.76 ± 0.01 (36)

[a] Data are expressed as mg g^{-1} of fresh weight. Values are the mean ± SD (n = 3). The figures in parentheses represent caffeine levels expressed as a percentage of the amount detected in *C. arabica*.

RESULTS

Endogenous Purine Alkaloids in *Coffea* Leaves. The levels of caffeine in *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* in young and mature leaves were 13−36% of those found in *C. arabica*. In all four species, young leaves contained higher concentrations of caffeine than mature leaves (Table 1). Theobromine, which was not detected in extracts from *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*, was present in young and mature leaves of *C. arabica* at concentrations of 1.8 and 0.1 mg g^{-1} (fw), respectively. No other endogenous purine alkaloids were detected in any of the leaf extracts.

Metabolism of [8-^{14}C]Adenine. As plants readily convert adenine to AMP, isotopically labeled adenine can be used to investigate the AMP-derived caffeine biosynthetic pathway (Suzuki et al., 1992) (see Figure 1). Table 2 shows results of the overall metabolism of [8-^{14}C]adenine. There was little difference in the incorporation of label into nucleotides in the four *Coffea* species, although the level of radioactivity associated with theobromine and caffeine was much higher in young leaves of *C. arabica* than it was in *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*. In *C. arabica* 46.7% of the radioactivity taken up by the leaf segments was incorporated into the two purine alkaloids; the figures for *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* were 15.0, 0.4, and 0.4%, respectively. Extensive labeling of the purine catabolites, allantoin, allantoic acid, and CO_2, was observed only in the low-caffeine-containing *Coffea* plants. In *C. eugenioides*, More >30% of total radioactivity was released as $^{14}CO_2$, whereas the ureides, allantoin, and allantoic acid were the most heavily labeled compounds in *C. bengalensis*. Thus, in *C. arabica*, [8-^{14}C]adenine is metabolized preferentially to 7-methylxanthine and converted to caffeine via theobromine, whereas in the low-caffeine-containing *Coffea* species, it appears to be converted primarily to xanthine and enters the purine catabolism pathway.

Catabolism of [8-^{14}C]Caffeine. Data on the catabolism of [8-^{14}C]caffeine by young and mature leaves from *C. salvatrix*, *C. eugenioides*, and *C. bengalensis* are presented in Table 3. Little or no catabolism occurred in leaves of *C. salvatrix* and *C. bengalensis*. Similar results were obtained in an earlier study with *C. arabica* leaves (Ashihara et al., 1996b). In contrast, very extensive catabolism of [8-^{14}C]caffeine was observed in leaf

Table 2. Metabolism of [8-^{14}C]Adenine by Young Coffee Leaves[a]

metabolite	*C. salvatrix*	*C. eugenioides*	*C. bengalensis*	*C. arabica*
methanol-soluble	55.4 ± 1.3	37.4 ± 0.0	64.3 ± 1.3	63.2 ± 1.0
nucleotides	8.2 ± 1.6	20.4 ± 1.5	6.9 ± 0.4	4.4 ± 0.5[b]
adenine	2.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	
theobromine	12.3 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	26.2 ± 3.2
caffeine	2.7 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	20.5 ± 3.5
xanthine	7.1 ± 1.2	3.3 ± 0.1	3.0 ± 0.3	8.4 ± 1.7
allantoin	10.2 ± 1.2	18.5 ± 0.5	33.8 ± 1.4	
allantoic acid	10.2 ± 0.2	5.1 ± 0.4	6.6 ± 0.5	
unknown	2.1 ± 0.1	1.7 ± 0.4	4.6 ± 0.3	
CO_2	17.6 ± 1.1	31.9 ± 2.3	5.4 ± 1.0	3.2 ± 0.2
methanol-insoluble	27.0 ± 0.2	30.8 ± 2.4	30.3 ± 2.3	35.0 ± 0.7
total uptake (kBq)	83.4 ± 6.0	189.9 ± 16.6	155.0 ± 13.4	123.0 ± 3.0

[a] Segments of young leaves were incubated with 9.4 μM [8-^{14}C]adenine for 18 h. Values for *C. arabica* were taken from Ashihara et al. (1996a). Incorporation of radioactivity into metabolites is expressed as percentage of total uptake ± SD ($n = 3$). Total uptake of radioactivity is expressed as kBq g^{-1} of leaf (fresh weight). [b] This value includes incorporation into nucleotides, allantoin, allantoic acid, adenine, and unidentified methanol-soluble compounds.

Table 3. Metabolism of [8-^{14}C]Caffeine by Young and Mature Coffee Leaves[a]

species	leaves	distribution of recovered radioactivity (% of total ± SD)								total uptake of radioactivity (kBq ± SD)
		Cf	Tp	3-mX	1-mX	X	ureides	urea	CO_2	
C. salvatrix	young	100 ± 0.0	nd[b]	nd	nd	nd	nd	nd	nd	25.3 ± 2.9
	mature	99.7 ± 0.1	nd	nd	nd	nd	nd	nd	0.3 ± 0.1	45.2 ± 0.1
C. eugenioides	young	23.6 ± 2.5	37.6 ± 1.3	21.6 ± 0.1	9.1 ± 0.5	1.8 ± 0.2	0.6 ± 0.5	2.0 ± 0.1	0.8 ± 0.0	91.5 ± 3.2
	mature	18.5 ± 0.7	14.3 ± 0.9	29.7 ± 1.4	11.6 ± 0.3	4.1 ± 0.0	1.5 ± 0.1	4.5 ± 0.1	13.5 ± 1.8	80.9 ± 7.2
C. bengalensis	young	100 ± 0.0	nd	nd	nd	nd	nd	nd	nd	49.1 ± 6.4
	mature	96.5 ± 1.0	3.3 ± 1.0	nd	nd	nd	nd	nd	0.2 ± 0.1	42.8 ± 3.2

[a] Segments of young leaves were incubated with 8.9 μM [8-^{14}C]caffeine for 18 h. Incorporation of radioactivity into metabolites is expressed as percentage of total uptake ± SD ($n = 3$). Total uptake of radioactivity is expressed as kBq g^{-1} of leaf (fw). Caffeine (Cf), theophylline (Tp), 3-methylxanthine (3-mX), 1-methylxanthine (1-mX), xanthine (X). [b] nd, not detected.

Table 4. Metabolism of [8-^{14}C]Caffeine in a Pulse−Chase Experiment with Mature Leaves of *C. eugenioides*[a]

metabolite	4 h (pulse)	8 h (chase)	24 h (chase)
residual caffeine	52.0 ± 0.1	4.9 ± 0.1	3.4 ± 0.1
theophylline	21.4 ± 0.9	24.2 ± 0.6	8.1 ± 0.5
3-methylxanthine	16.0 ± 0.1	44.0 ± 0.2	46.9 ± 1.9
1-methylxanthine	6.1 ± 0.6	6.6 ± 0.4	7.4 ± 0.0
xanthine	nd[b]	3.9 ± 0.4	5.8 ± 0.2
allantoin	0.9 ± 0.1	18 ± 0.0	1.4 ± 1.1
allantoic acid	1.1 ± 0.0	2.5 ± 0.0	nd
urea	1.2 ± 0.1	3.5 ± 0.1	2.9 ± 0.1
CO_2	1.3 ± 0.2	8.3 ± 0.9	24.2 ± 2.0

[a] Leaf segments (100 mg of fresh weight) were incubated with 18 μM [8-^{14}C]caffeine for 4 h (pulse), and then the incubation medium was replaced by fresh medium without tracer. The radioactivity was "chased" for a further 4 and 20 h. Incorporation of radioactivity into each compound is expressed as a percentage of the total radioactivity recovered. Mean values ± SD ($n = 3$) shown. Total radioactivity taken up by the tissues was 31.2 ± 4.0 kBq g^{-1} of leaf. [b] nd, not detected.

segments from *C. eugenioides*. More than 75% of [8-^{14}C]-caffeine taken up by the segments was catabolized in both young and mature *C. eugenioides* leaves, with radioactivity recovered as theophylline, 3-methylxanthine, 1-methylxanthine, xanthine, ureides, urea, and CO_2 This indicates that [8-^{14}C]caffeine undergoes demethylation, probably via the routes indicated in Figure 2, resulting in the production of xanthine, which is degraded to CO_2 and NH_3 via the conventional purine catabolic pathway.

The rate of uptake of [8-^{14}C]caffeine by leaf segments of *C. eugenioides* was much higher than those by the segments of *C. salvatrix* and *C. bengalensis* (Table 3). A similar trend was also observed when [8-^{14}C]theophylline was used as a precursor (see Table 5). The reasons for this are unclear, although it is possible that passive transport along a concentration gradient, from the incubation medium into the cells of the leaf, is enhanced in keeping with the extent to which the absorbed purine alkaloid is catabolized.

To obtain further information on the pathway utilized for the catabolism of caffeine in *C. eugenioides*, pulse−chase experiments with [8-^{14}C]caffeine were carried out using mature leaves (Table 4). Caffeine, theophylline, and 3-methylxanthine were the most heavily labeled compounds after the 4 h pulse. The radioactivity associated with caffeine declined after the leaves were transferred to the nonradioactive medium. In contrast, ^{14}C-labeled theophylline, 3-methylxanthine, 1-methylxanthine, xanthine, ureides, urea, and CO_2 increased after the 4 h chase, with >40% of the radioactivity taken up during the pulse being incorporated into 3-methylxanthine. After a further 20 h chase, radioactivity associated with theophylline, ureides, and urea declined, whereas the level of ^{14}C associated with 3-methylxanthine, 1-methylxanthine, and xanthine changed little and $^{14}CO_2$ evolution increased from 8.3 to 24.2% of the recovered radioactivity.

Catabolism of [8-^{14}C]Theophylline. *C. arabica* leaves catabolize theophylline much more rapidly than caffeine (Ashihara et al., 1996b). It was, therefore, of interest to investigate the fate of theophylline when incubated with young and mature leaves of *C. salvatrix*, *C. eugenioides*, and *C. bengalensis*. The data obtained are presented in Table 5. [8-^{14}C]Theophylline was converted to a range of catabolites, most extensively by *C. eugenioides* with the evolution of $^{14}CO_2$ from mature leaves being 3 times greater than that from young leaves, from which more than half of radioactivity was recovered as 3-methylxanthine. More label was associated with 3-methylxanthine than 1-methylxanthine, indicating that the main route for catabolism of theo-

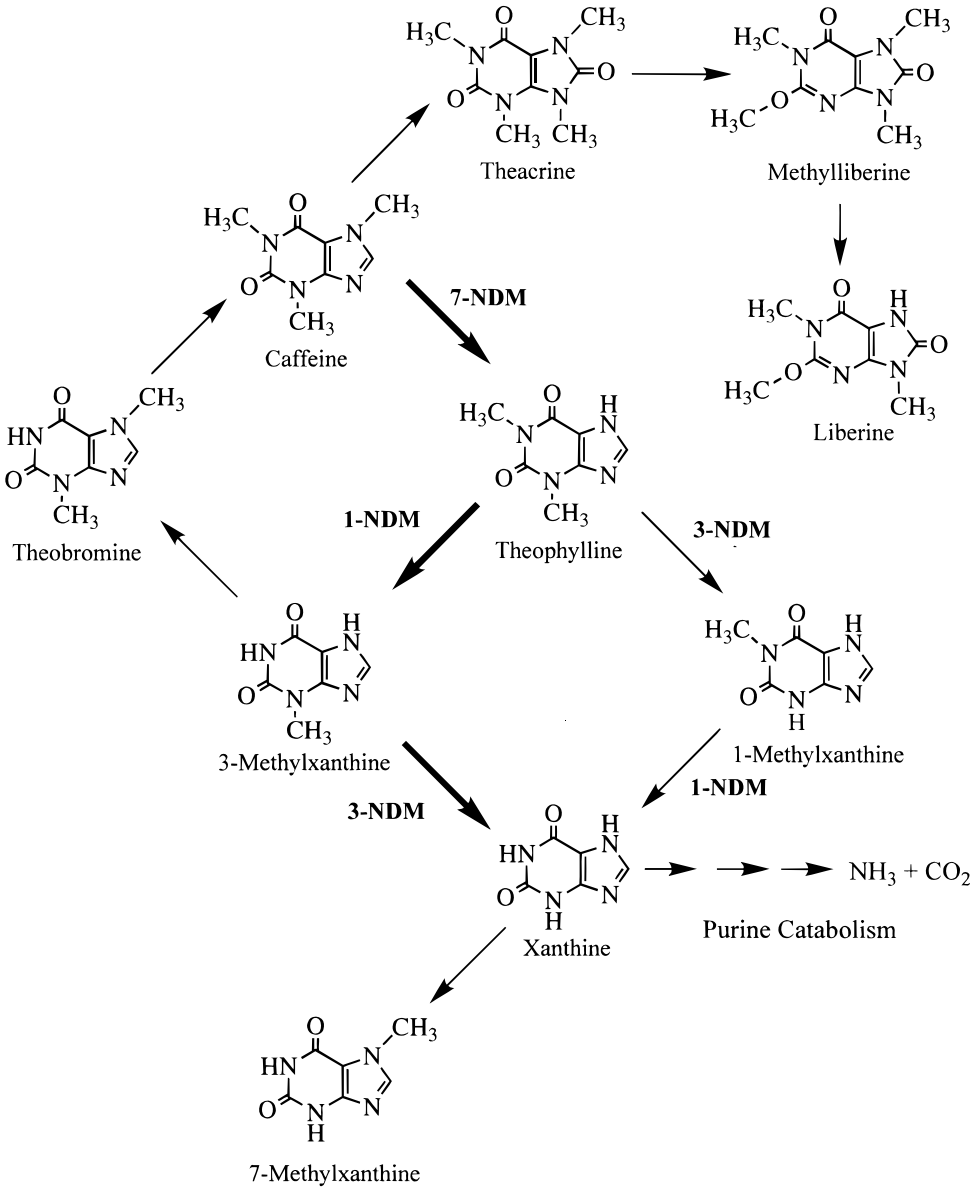

Figure 2. Caffeine and purine catabolic pathways in *Coffea* plants. Bold arrows indicate the main pathway via which caffeine is degraded in *C. eugenioides.* The conversion of caffeine to theophylline is effectively blocked in *C. arabica* as well as in *C. salvatrix* and *C. bengalensis* in which the catabolism of theophylline is also slow. In *C. arabica,* theophylline is catabolized to xanthine, traces of which are converted to 7-methylxanthine, but most enters the purine catabolism pathway and is degraded to CO_2 and NH_3. In *C. dewevrei*, *C. liberica*, and *C. abeokutae*, but not the other species referred to above, caffeine is converted to theacrine, methylliberine, and liberine (Baumann et al., 1976; Petermann and Baumann, 1983). In tea leaves, the conversion of caffeine to theophylline is blocked, whereas theophylline is also demethylated to xanthine as shown in *Coffea* plants, but there is also resynthesis of caffeine via a theophylline → 3-methylxanthine → theobromine → caffeine salvage pathway (Ashihara et al., 1997). Enzymes: *N-1* demethylase (1-NDM), *N-3* demethylase (3-NDM), and *N-7* demethylase (7-NDM). To date, there are no reports on the preparation of cell-free extracts containing the *N*-demethylase activities involved in the catabolism of purine alkaloids in coffee and tea.

phylline to xanthine is via 3-methylxanthine in *C. eugenioides.* Metabolism of [8-^{14}C]theophylline was slower in *C. bengalensis* with proportionally more radioactivity being recovered as unmetabolized theophylline and less as $^{14}CO_2$ and intermediates of purine catabolism. There was relatively little catabolism of [8-^{14}C]theophylline by leaves of *C. salvatrix*, with >90% of the total radioactivity taken up in leaves being the unmetabolized substrate and only minimal incorporation of label into purine catabolites.

DISCUSSION

The biosynthesis of caffeine in coffee has been investigated primarily in *C. arabica* (Looser et al., 1974; Baumann et al., 1978; Roberts and Waller, 1979; Baumann and Gabriel, 1984; Suzuki and Waller, 1984a,b; Negishi et al., 1985a; Fujimori and Ashihara, 1994; Ashihara et al., 1996a,b; Mosli Waldhauser et al., 1997; Crozier et al., 1998; Ashihara and Crozier, 1999), and the route illustrated in Figure 1 also operates in tea (*Camellia sinensis*) (Suzuki and Takahashi, 1976; Negishi et al., 1985b; Ashihara and Kubota, 1986; Fujimori et al., 1991; Negishi et al., 1992; Ashihara et al., 1995, 1997; Ito and Ashihara, 1999) Although studied in less detail, the main biosynthetic pathway from purine nucleotides to theobromine and/or caffeine appears to be similar, if not identical, in other species of *Coffea* (Mazzafera et al., 1994) and *Camellia irrawadiensis*

Table 5. Metabolism of [8-^{14}C]Theophylline by Young and Mature Coffee Leaves[a]

species	leaves	distribution of recovered radioactivity (% total ± SD)								total uptake of radioactivity (kBq ± SD)
		Tp	3-mX	1-mX	X	Alln	Alla	urea	CO_2	
C. salvatrix	young	99.5 ± 0.0	nd[b]	nd	nd	nd	nd	nd	0.5 ± 0.0	54.8 ± 12.2
	mature	95.2 ± 0.7	2.2 ± 0.2	nd	0.5 ± 0.1	nd	nd	0.5 ± 0.0	1.0 ± 0.4	50.2 ± 5.2
C. eugenioides	young	26.6 ± 1.0	53.8 ± 1.0	0.8 ± 0.1	1.3 ± 0.2	4.8 ± 0.7	1.0 ± 0.0	3.3 ± 0.1	7.9 ± 2.9	86.3 ± 14.2
	mature	36.4 ± 0.3	26.2 ± 0.7	0.8 ± 0.0	4.8 ± 0.3	0.8 ± 0.1	1.3 ± 0.0	3.5 ± 0.3	23.5 ± 0.7	85.7 ± 1.5
C. bengalensis	young	82.4 ± 1.0	6.9 ± 0.0	2.0 ± 0.4	nd	3.4 ± 0.2	nd	nd	1.3 ± 0.4	41.7 ± 1.8
	mature	78.2 ± 0.3	14.2 ± 0.2	3.1 ± 0.2	nd	3.7 ± 0.4	nd	nd	0.9 ± 0.1	34.5 ± 3.6

[a] Segments of young leaves were incubated with 9.1 μM [8-^{14}C]theophylline for 18 h. Incorporation of radioactivity into metabolites is expressed as percentage of total uptake ± SD ($n = 3$). Total uptake of radioactivity is expressed as kBq g^{-1} of leaf (fw). Theophylline (Tp), 3-methylxanthine (3-mX), 1-methylxanthine (1-mX), xanthine (X), allantoin (Alln), allantoic acid (Alla). [b] nd, not detected.

(Ashihara and Kubota, 1987) as well as maté (*Ilex paraguariensis*) (Ashihara, 1993), cocoa tea (*Camellia ptilophylla*) (Ashihara et al., 1998), and guaraná (*Paullinia cupana*) (H. Ashihara and T. W. Baumann, unpublished data).

In the present study, the biosynthesis and catabolism of caffeine were investigated in *C. eugenioides*, *C. salvatrix*, and *C. bengalensis*, three low-caffeine-containing species of coffee. The data from feeds with [8-^{14}C]adenine demonstrate that, as in *C. arabica* (Suzuki et al., 1992; Ashihara et al., 1996a; Crozier et al., 1997), caffeine is synthesized from purine nucleotides in young leaves of all three *Coffea* species However, the rate of caffeine biosynthesis is much lower than in *C. arabica*, especially in *C. bengalensis*, and this is one reason for their low caffeine content.

The size of endogenous caffeine pools can also be regulated by the rate of caffeine catabolism, further methylation, and oxidation, and there is some diversity in the pathways operating in different species of coffee (Mazzafera et al., 1991, 1994). For example, leaves of *C. dewevrei*, *C. liberica*, and *C. abeokutae* convert caffeine to theacrine (1,3,7,9-tetramethyluric acid), methylliberine [*O*(2)1,7,9-tetramethyluric acid], liberine [*O*(2)1,9-trimethyluric acid] and other methyluric acids (Baumann et al., 1976; Petermann and Baumann, 1983), whereas the main route in *C. arabica* is caffeine → theophylline → 3-methylxanthine → xanthine → uric acid → allantoin → allantoic acid → → $CO_2 + NH_3$ (see Figure 2). The reaction velocity of the initial step in this pathway, the conversion of caffeine to theophylline, is very low and, as a consequence, caffeine accumulates in *C. arabica* (Ashihara et al., 1996b). The present study revealed that among three low-caffeine-containing *Coffea* species, only *C. eugenioides* possessed a strong capacity for caffeine catabolism, with the demethylation of theophylline to xanthine proceeding mainly via 3-methylxanthine and to a lesser extent via 1-methylxanthine (see Figure 2). The reduced caffeine content of *C. eugenioides* is, thus, the result of a low rate of caffeine biosynthesis combined with a high rate of caffeine catabolism. In *C. salvatrix* and *C. bengalensis*, catabolism of both caffeine and theophylline is very slow; therefore, the low caffeine content of these *Coffea* species appears to be due primarily to their reduced biosynthetic activity.

7-Methylxanthine, produced via xanthine, is a minor catabolite of [8-^{14}C]theophylline in *C. arabica* leaves (Ashihara et al., 1996b), whereas in tea and maté leaves small amounts of theophylline are salvaged for the synthesis of caffeine via a theophylline → 3-methylxanthine → theobromine → caffeine pathway (Figure 2) (Ashihara et al., 1997; Ito et al., 1997). In the present study with *C. eugenioides*, *C. salvatrix*, and *C. bengalensis*, [8-^{14}C]theophylline was neither converted to 7-methylxanthine nor salvaged for the synthesis of caffeine. It has been reported previously that 7-methylxanthine is not involved in caffeine catabolism in *C. dewevrei* (Mazzafera, 1993). Unlike *C. dewevrei*, and *C. liberica*, and *C. abeokutae*, there is no evidence that *C. arabica*, *C. eugenioides*, *C. salvatrix*, and *C. bengalensis* convert caffeine to theacrine, methylliberine, and liberine.

The results of the present study are useful for the long-term aims of using biotechnology to produce transgenic, caffeine-deficient *C. arabica* plants. *C. eugenioides* degrades caffeine via theophylline and so, unlike the other species, contains a specific 7*N*-demethylase activity. The substrate specificity of this demethylase seems to be different from that of the *N*-demethylase isolated from bacteria, such as *Pseudomonas putida* and *Pseudomonas cepacia* (Ashihara and Crozier, 1999), which catabolizes caffeine to theobromine rather than theophylline (Asano et al., 1994). Expression of the bacterial demethylase in *C. arabica* is unlikely to result in caffeine deficiency as caffeine will be degraded to theobromine, which is the immediate precursor of caffeine in coffee. In contrast, insertion of the 7*N*-demethylase encoding gene from *C. eugenioides* into the genome of *C. arabica* is much more likely to produce caffeine deficiency because the *eugenioides* gene product will catalyze the conversion to caffeine to theophylline and the native Arabica enzymes have the capacity to rapidly degrade theophylline. Work on the isolation and characterization of this novel *N*-demethylase from *C. eugenioides* is in progress.

ACKNOWLEDGMENT

This research was supported by the Royal Society, which provided H.A. and A.C. with Japan−U.K. travel grants. Part of this work is also supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 10640627) to H.A. We thank Dr. Maro Söndahl (Fitolink Corp., Mount Laurel, NJ) for his help in obtaining seeds of the four species of coffee used in this study from the Instituto Agronómico, Campinas, S.P., Brazil.

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Received for review November 5, 1998. Revised manuscript received May 18, 1999. Accepted June 2, 1999.

JF981209N